

The X-ray structure of NCAM Ig1-2-3 was determined to 2.0 Å resolution (see Table 1 of Figure 1). In the structure of Ig1-2-3, the Ig1 and Ig2 modules are positioned in an extended conformation with Ig3 oriented at an angle of approximately 45° to the Ig1-Ig2 axis (Figure 3). The linker regions between Ig1-Ig2 and between Ig2-Ig3 are short and comprise only two (Lys98 – Leu99) and one (Asn190) residues, respectively. The overall structure of the Ig1 and Ig2 modules is very similar to the previously determined Ig1-2 structure (Kasper et al., 2000) with root mean square deviations (r.m.s.d.) of 0.7 (96 Cα atoms) and 0.8 Å (93 Cα atoms), respectively. In the Ig1-2-3 structure, the tilt angle between Ig1 and Ig2 is 11° and thereby differs by 13° compared to the Ig1-2 structure.

The 98-residue Ig3 module of rat NCAM adopts the topology of an intermediate type 1 (I1) set Ig module (Casasnovas et al., 1998). In the Ig3 module, the classical β-sandwich consists of two β-sheets with a total of nine β-strands (Figure 3B). The A, B, D, and E β-strands make up one sheet and the A', C, C', F, and G β-strands the second sheet. A cysteine bridge Cys216 – Cys269 connects the two β-sheets. All strands are anti-parallel except for the A' strand, which runs parallel to the C-terminal part of the G strand. Ig3 contains one site for N-linked glycosylation at Asn203 positioned in the A' strand. The E-F loop (residues Lys261 – Asp263) forms a 3<sub>10</sub> α-helical turn. The overall structure of rat Ig3 is similar to the structure of chicken Ig3 (Atkins et al., 2001) with r.m.s.d. of 1.65 Å (95 Cα atoms).

#### Parallel interactions between Ig modules

Several characteristic interactions are observed in the structure of the NCAM Ig1-2-3 fragment which may be divided into two groups: Interactions where the long axes (N- to C-terminus) of two interacting Ig1-2-3 molecules are oriented in a parallel manner and interactions where the long axes are oriented in an anti-parallel manner. One parallel interaction and three major anti-parallel interactions are observed in the crystal.

The parallel, cross-like dimer interaction of NCAM Ig1-2-3 involves the Ig1 and Ig2 modules (Figure 5). The total buried surface area of this interface is 1594 Å<sup>2</sup> (per dimer), which is similar to that previously observed in the Ig1-2 cross-like dimers (Kasper et al., 2000). The most prominent feature of the Ig1-to-Ig2 interaction is the intercalation of two aromatic residues of Ig1, Phe19 and Tyr65, into hydrophobic pockets formed by Ig2 residues (Figure 5A), which was also observed in the Ig1-2 structure. However, a tighter Ig1 to Ig2 binding interface is observed in the Ig1-2-3

structure, where the hydroxyl group of Tyr65 forms a direct hydrogen bond (H-bond) with Glu171, instead of a water-mediated H-bond as observed in Ig1-2. Tyr65 also makes three H-bonds to the side chains of Lys133, Glu171, and Arg173. Arg173 forms part of the Ig2 hydrophobic pocket and makes two H-bonds to Thr63. The parallel orientation of the Arg173 and Phe19 side chains and the distance between the N $\alpha$  atom of the guanidinium group of Arg173 and the C $\zeta$  atom of the benzene ring of Phe19 (3.4 Å) suggest a cation- $\pi$  interaction between these two residues (Flocco and Mowbray, 1994).

Dynamic Light Scattering (DLS) measurements showed that deglycosylated Ig1-2-3 forms a single species of molecules in solution with a molecular weight of ~78 kDa, corresponding to a dimer. In order to demonstrate that Ig1-2-3 dimerization is mediated by the observed Ig1 to Ig2 binding, we produced a mutant of Ig1-2-3 (Ig1-2-3mut) containing three Ala substitutions: E11A, E16A, and K18A. These mutations have previously been shown to completely abolish dimerization of the Ig1-2 NCAM fragment in solution (Jensen et al., 1999). In the present structure Glu11 and Glu16 form intramolecular salt bridges, respectively, with Arg177 and Lys98 from the Ig1 to Ig2 linker region (not shown). These salt bridges probably contribute to the proper orientation of Ig1 with respect to Ig2 and therefore are important for the Ig1-to-Ig2 interaction. Lys18 forms an H-bond with the carboxyl group of Arg177 from the Ig2 module stabilizing the Ig1-Ig2 interaction (Figure 5A). Lys18 is located near Phe19, which is the critical residue for the Ig1-to-Ig2 interaction as it was clearly demonstrated earlier (Atkins et al., 2001). Therefore, disruption of the Lys18 - Arg177 H-bond may affect the orientation of Phe19 leading to elimination of the Ig1-to-Ig2 interaction. The molecular weight of the Ig1-2-3mut fragment was determined by DLS to be ~34 kDa, indicating a monomer. This confirms that Ig1-2-3 dimerization is mediated by Ig1-to-Ig2 binding.

Parallel (*cis*) interactions are not uncommon among cell adhesion molecules. Thus, *cis* dimerization has been demonstrated for the cell adhesion molecules C-CAM1, C-CAM2, ICAM-1, nectin-2 $\alpha$ , and JAM belonging to the Ig superfamily (Hunter et al., 1996; Casasnovas et al., 1998; Miyahara et al., 2000; Kostrewa et al., 2001) as well as for N-, E-, and C- cadherins (Shapiro et al., 1995; Takeda et al., 1999; Brieher et al., 1996). It was shown that the dimeric form of C-cadherin is capable of adhesion, whereas the monomeric form is not (Brieher et al., 1996).

### Anti-parallel interactions between Ig modules

An anti-parallel interaction takes place between the Ig2 and Ig3 modules of two Ig1-2-3 molecules, thereby forming arrays of Ig1-2-3 dimers (Figure 4A,B). Ig2 of one molecule binds to Ig3 of a second molecule, and *vice versa* (Figure 3B). The residues involved are 112-115, 143-146, and 158-161 from the B-strand, CD-loop/D-strand, and E-strand of Ig2, and residues 200-205, 261, and 278-289 from the A'-strand, EF-loop, and G-strand of Ig3. A central element of this interaction is the intercalation of the side chain of Phe287 from Ig3 into a hydrophobic pocket formed by the side chains of Val145, Arg146, and Arg158 of the Ig2 module and Lys285 from Ig3. Arg158 is also involved in water-mediated hydrogen bonding to residues Lys261 and Ala288, and Gly159 makes a direct H-bond to Asn203.

The crystal packing leaves room for glycosylation at Asn203. In order to accommodate N-linked glycosylation at this site, the side chain of Asn203 has to adopt another rotamer conformation. Thereby, the carbohydrate will point away from the binding site and towards a solvent channel in the crystal, and consequently Asn203 will not interfere with Ig2-Ig3 interactions. An interaction between the two Ig3 modules is observed at the interface, as Gln196 makes a water-mediated H-bond with Gln278. The total buried surface of the Ig2-to-Ig3 interface is 1407 Å<sup>2</sup> per dimer. According to Janin (1997), the probability of finding a non-specific interface of the size of the Ig2-to-Ig3 contact is only 1.9%.

Another anti-parallel interaction between two Ig1-2-3 molecules is formed between two Ig2 modules (Figure 4C,D). This interaction involves residues 103-121 and 150-158 of the AA'-loop/A'-strand/A'B-loop and the DE-loop/E-strand and has the total buried surface of 958 Å<sup>2</sup> per dimer (Figure 4C). Here, the central residue appears to be Glu114, which makes two H-bonds to Ser151 (side chain and backbone). Apart from an extensive hydrogen-bonding network, especially through water molecules, Val117, Val119, Leu150, and Tyr154 of both Ig2 modules form a number of hydrophobic contacts with each other at the Ig2-to-Ig2 interface (not shown).

A slightly smaller anti-parallel interaction (858 Å<sup>2</sup> of total buried surface per dimer) is formed between the Ig1 and Ig3 modules (Figure 4C,D), involving residues 32-47 and 76-88 from the C-strand/CC'-loop/C'-strand/C'D-loop and F-strand/FG-loop/G-strand in Ig1, and residues 198, 213-223, and 248-253 from the A-strand, B-strand/BC-loop, and D-strand/DE-loop in Ig3 (Figure 5D). Arg198 and Asp249 form direct H-bonds to the backbone oxygen atoms of Ala81 and Glu82 and two salt bridges with Lys76, respectively. Additionally, one water-mediated H-bond is formed between Lys42 and Asp250, one between Ser44 and Gly220, and two between

Ser44 and Glu223. The conserved Phe36 and Phe221 are packed against Asp249 and Gln47, respectively. Together two Ig1-to-Ig3 interaction sites and one Ig2-to-Ig2 site make up a predominant contact between Ig1-2-3 dimers in the crystal ( $2654 \text{ \AA}^2$ ) forming the second array of Ig1-2-3 dimers (Figure 4C,D) perpendicular to the Ig2-to-Ig3-mediated array (Figure 2A,B). Contact areas of similar sizes have been found in other CAMs. *Cis* dimers of human ICAM-1 and mouse JAM have  $1100 \text{ \AA}^2$  and  $1200 \text{ \AA}^2$  of total buried surface area (per dimer), respectively (Casasnovas et al. 1998; Kostrewa et al., 2001), whereas *trans* dimers of rat CD2 and chicken axonin-1/TAG-1 have even larger contact areas of  $1300 \text{ \AA}^2$  and  $2000 \text{ \AA}^2$  (Jones et al., 1992; Freigang et al., 2000).

### **Ig3 inhibits NCAM-dependent neurite outgrowth**

NCAM-NCAM interaction is known to induce neurite outgrowth from NCAM-expressing PC12-E2 cells grown on a confluent monolayer of NCAM-expressing fibroblasts (Kolkova et al., 2000). Inhibition of the NCAM-NCAM interaction will therefore inhibit neurite outgrowth in PC12-E2 cells.

In order to examine the biological significance of the observed Ig1-to-Ig3 and Ig2-to-Ig3 contacts in the structure of NCAM Ig1-2-3, we tested the inhibitory effect of the recombinant Ig3 module on NCAM-NCAM adhesion. Furthermore, we prepared two Ig3 mutants containing mutations of the residues R198A, D249G, E253A (Ig3mut1) of the Ig1-to-Ig3 contact site (see Figure 5D) and K285A, F287A (Ig3mut2) of the Ig2-to-Ig3 contact site (see Figure 5B). In Figure 4 it can be seen that the wildtype Ig3 module (Ig3wt) indeed has an inhibitory effect, whereas both mutants are inactive, thereby strongly supporting that both the Ig1-to-Ig3 and Ig2-to-Ig3 contact sites are participating in homophilic interactions.

A similar co-culture test-system of NCAM-expressing chicken retinal ganglion cells grown on top of NCAM-140-transfected mouse L-cells has been successfully used to demonstrate a disruptive effect of mutations in the Ig3 module homophilic binding site (Ig1-to-Ig3 binding site in the present work) as well as to show an inhibition of neurite outgrowth by synthetic peptides representing this homophilic binding site (Sandig et al. 1994).

### **Interaction interface peptides inhibit neurite outgrowth**

It has previously been demonstrated that peptides representing homophilic binding sequences from Ig3 and Ig2 modules of NCAM inhibit NCAM-mediated cell